NATIONAL AERONAUTICS AND SPACE ADMINISTRATION

GENETIC STUDIES OF HYDROGEN BACTERIA AND THEIR APPLICATIONS TO BIOLOGICAL LIFE SUPPORT SYSTEMS

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I Studies with Hydrogenomonas eutropha Bacteriophages

The discovery of bacteriophages active against H. eutropha has demonstrated that the possibility of a phage infection of this bacterium in a life-support system must be considered. The important questions then become

1. What would be the effect of such an infection in a continuously growing culture?
2. Can steps be taken to reduce the possibility of infection?

One would expect the answer to question 1 to depend upon the growth conditions employed and the characteristics of the infecting phage. Under a given set of growth conditions the infectious pattern displayed by the infecting phage would be of prime importance. However, since we are dealing with a number of phage types (four types have been isolated to date) we must determine whether their infectious patterns are similar or different before the effect of an infection can be predicted. Once the range of effects is known, possible control or preventive measures can be explored.

One way of measuring the relatedness of different phage types is by isolating phage-resistant mutants of H. eutropha and determining their cross-resistance to the various phages. Thus H. eutropha cultures resistant to each of the four phage types were obtained by exposing a large bacterial population to a large number of phages on heterotrophic agar. Phage-resistant clones were then isolated and tested for their sensitivity to the other three phage types. The results of these tests are shown in Table 1. The data suggest that phage types HeMa and HeMb are closely related, as might be expected since both phages were isolated from nearby sources in Maryland and behaved similarly in many respects. Types HeW and HeC also appear to be related which is somewhat surprising since HeW originated in Washington, D.C. and HeC came from California.

Phage types HeC and HeW are more virulent for H. eutropha than are HeMa and HeMb. Cultures of autotrophically growing H. eutropha were therefore infected with types HeC and HeW phages and examined for clearing as an indication of cell lysis. (Table 2). Culture #1

TABLE 1

CROSS RESISTANCE PATTERN AMONG *H. EUTROPHA* BACTERIOPHAGES

Culture		phage Type Tested			
		Hema	Hemb	HeC	Hew
H. eutropha resistant to:	Hema	−	−	+	+
:	Hemb	−	−	+	+
:	HeC	+	+	−	−
:	Hew	+	+	−	−

\+ = lysis
− = no lysis

TABLE 2

EFFECT OF PHAGE INFECTION
DURING AUTOTROPHIC GROWTH OF H. EUTROPHA

	OD 540	
Phage Type	0 hrs.	22 hrs.
—	0.10	0.66
HeC	0.10	0.17
Hew	0.10	0.07

was an uninfected control and increased in turbidity from O.D. 0.1 to 0.66 during the 22 hr. incubation period. Culture #2 infected with phage HeC and culture #3 infected with phage HeW showed a definite impairment of growth during the same period. The phage used for these experiments, however, were suspended in 15 g/l tryptic soy broth and since 0.1 ml of phage suspension was added to 10 ml of culture, there was a small amount of organic matter present in each flask, which may have enhanced phage virulence. This suggests that phage virulence might also be enhanced by the organic matter present in urine - supplemented media, as suggested for a life support system.

II Growth of H. eutropha Under Simultaneous Autotrophic and Heterotrophic Conditions

The efficient cultivation of Hydrogen Bacteria in a urine-supplemented medium under an atmosphere of H_2, O_2 and CO_2 requires a knowledge of the metabolism of the bacterium under these conditions. The high organic content of urine necessitates a study of the effect of organic compounds on the autotrophic processes of the culture. The most obvious effects to be investigated would be with regard to the activities of the hydrogen utilizing (hydrogenase) and the carbon dioxide fixing systems. We have concerned ourselves mainly with hydrogenase activity to date.

We previously reported that the hydrogenase system of H. eutropha is at least partially active in the presence of a number of organic compounds and is fully active when H_2, O_2 and CO_2 are present along with an organic compound. Growth was compared when cells were incubated in 0.4% glutamate under air and 0.4% glutamate under H_2, O_2 and CO_2. The results consistently showed an early growth inhibition when H_2, O_2 and CO_2 were present in addition to glutamate. After this early inhibition, growth in glutamate plus the gas mixture proceeded at the same rate as the heterotrophic culture in glutamate alone. Thus it appeared as if the presence of the autotrophic gas mixture inhibited the utilization of glutamate.

To study the effect in detail two flasks containing 0.4% glutamate in Bongers' salts were inoculated to low turbidity . One culture was incubated in air and the other under 70% H_2, 20% O_2 and 10% CO_2. The optical densities of the cultures were recorded at frequent intervals during the early stages of growth. The results can be seen in Table 3. At 3.75 hours the ratio of growth in the glutamate plus H_2, O_2 CO_2 flask compared to the heterotrophic flask is .07/0.11 or about 2/3 and this ratio holds for all the readings during exponential growth. This effect is extremely reproducible; in every case the density of the culture under the autotrophic atmosphere was about 66% of the culture under air from four hours post-inoculation throughout the exponential growth period. This means that within the first four hours of incubation the culture under simultaneous conditions is inhibited for a period equal to 2/3 of a generation cycle. In 0.4% glutamate under air at 30C the generation time of H. eutropha is about two hours. The inhibition period therefore extends for roughly 1-1/3 hours. The multiplication factors seen in Table 3 demonstrate that little growth and no inhibition occurs within the first 1-1/2 hours incubation and that from 3-3/4 hours on the multiplication factors for the two cultures are very similar. Essentially all of the 1-1/3 hour inhibition period therefore occurs between 1-1/2 and 3-3/4 hours and this can be seen in the values for the multiplication factors during this period, which are 2.7 for the strictly heterotrophic culture and only 1.7 for the culture under mixed environment conditions.

Gottschalk[1] has reported an inhibition of the utilization of organic compounds by a hydrogenomonad similar to H. eutropha. The phenomenon observed by him, however, appears to be very different from the growth inhibition reported here. Gottschalk noticed a complete stoppage of growth in media containing fructose or glutamate when the gas phase was H_2 and O_2. He stated that the presence of H_2 repressed the induction of the heterotrophic catabolic system. If CO_2 was added to the gas mixture (to form the normal autotrophic almosphere) induction of the catabolic enzymes commenced. Thus H_2 was

1. Gottschalk, G. 1965. Biochem. Z. 341: 360-370.

TABLE 3

GROWTH OF H. EUTROPHA

UNDER AUTOTROPHIC, HETEROTROPHIC, AND SIMULTANEOUS CONDITIONS

Incubation Time (hrs)	0.4% Glutamate + air OD_{540} x Factor		0.4% Glutamate + H_2, O_2, N_2 OD_{540} x Factor		0.4% Glutamate + H_2, O_2, CO_2 OD_{540} x Factor		Salts + H_2, O_2, CO_2 OD_{540}
0	0.03		0.03		0.03		0.04
1.5	0.04		0.04		0.04		0.04
3.75	0.11	2.7	0.11	2.7	0.07	1.7	0.05
5.0	0.15	1.4	0.15	1.4	0.10	1.4	0.05
7.5	0.35	2.3	0.31	2.1	0.22	2.2	----
11.5	2.0		1.6		1.2		----
28.5	3.2		3.2		3.2		1.2

the repressor and CO_2 reversed the repression.

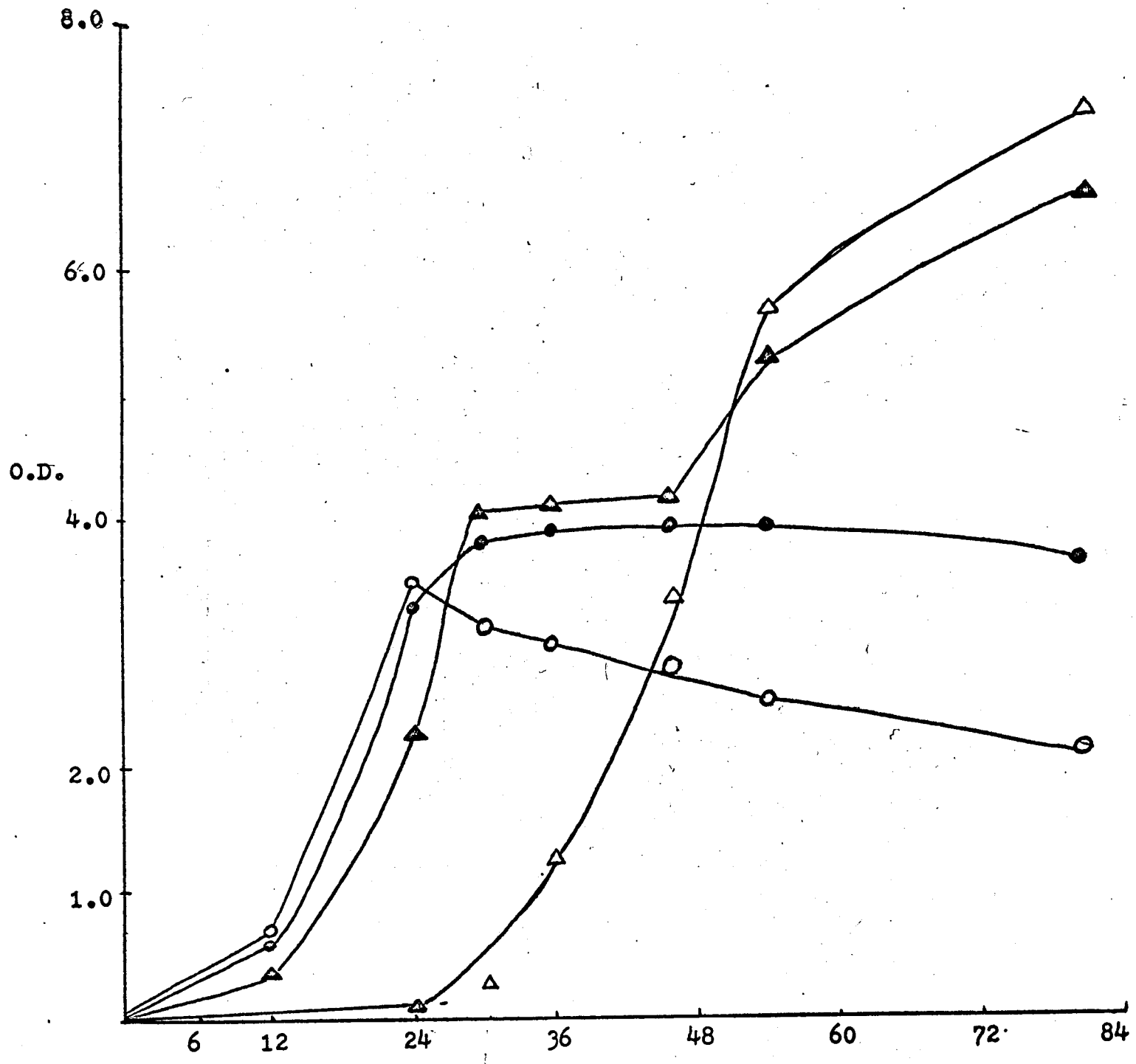
In our system with H. eutropha growth was never completely suppressed. Growth in glutamate medium incubated under an atmosphere of H_2, O_2 and N_2 was almost identical to that in air (Table 3). Thus hydrogen is not a repressor for glutamate utilization with H. eutropha nor does it cause a temporary inhibition of growth in the absence of CO_2. This means that CO_2 either alone or with H_2 is a temporary inhibitor of glutamate utilization.

Besides the temporary inhibition of growth during simultaneous autotrophic and heterotrophic conditions, several other aspects of growth under these conditions were revealed by extending the previously mentioned experiments over a longer time period. (Figure 1). The temporary inhibition when glutamate, H_2, O_2 and CO_2 are available can be seen as an increased lag. The growth curves are then parallel with all three atmospheres. The glutamate plus air and glutamate plus H_2, O_2, and N_2 flasks yield maxima at optical densities between 3.5 and 4.0 and glutamate measurements show that glutamate is exhausted when each culture reaches its peak. Under air the OD then decreases fairly rapidly, presumable due to cell autolysis. When a H_2, O_2 and N_2 mixture is present the OD remains quite constant near the peak level for a considerable time, suggesting that energy derived from H_2 oxidation can be used under starvation conditions to maintain cellular integrity.

The culture incubated under simultaneous conditions reaches a temporary maxima due to glutamate exhaustion at a cellular density slightly greater than the other glutamate cultures. Growth using both autotrophic and heterotrophic mechanisms simultaneously therefore does not occur to any great extent under these conditions. The temporary peak forms a plateau which extends for about 10 hours after which growth resumes autotrophically. This diphasic growth pattern is characteristic of the "diauxie" effect - the sequential utilization of one substrate and then another. The

FIGURE 1

GROWTH OF HYDROGENOMONAS EUTROPHA UNDER AUTOTROPHIC, HETEROTROPHIC, AND SIMULTANEOUS CONDITIONS.



○ = 0.4 % GLUTAMATE
○ = 0.4 % GLUTAMATE + 70% H_2, 20% O_2, 10% N_2
△ = 0.4 % GLUTAMATE + 70% H_2, 20% O_2, 10% CO_2
△ = AUTOTROPHIC (70% H_2, 20% O_2, 10% CO_2)

first substrate usually supports faster growth while the second is often catabolized adaptively; thus the plateau is usually the period required for induction of a catabolic system. In this case the substrate utilized first (glutamate) does support faster growth and the plateau period may be the time required for the induction of some component of the autotrophic system.

III Determination Of Nutritional Requirements of H. eutropha Auxotrophs

We have previously reported the isolation of 49 clones suspected of being nutritionally dependent or auxotrophic mutants of H. eutropha. We have now begun analyzing these mutants to determine their specific nutritional requirements. The clones were first checked for their ability to grow on glutamate-salts medium supplemented with either casamino acids or a vitamin mixture. None of the 49 clones showed evidence of a vitamin requirement alone. A number of the clones yielded growth on a medium supplemented with casamino acids. The specific amino acid required by each mutant culture was then determined by dividing the 20 common amino acids into four groups, determining which group satisfied the growth requirements for each culture, and then determining which amino acid in each group was required. Table 4 shows the amino acid auxotrophs and their respective requirements.

Strain A24 which requires both phenylalanine and tryptophan is in all likelihood blocked at a single point somewhere along the shikimic acid pathway. This pathway is common to the synthesis of both these amino acids and thus a single enzymatic block would lead to a requirement for both of these aromatic compounds.

The growth factor required by the remainder of the 49 cultures have not yet been identified. Several of these cultures have been tested for their ability to grow on a complex, defined tissue culture medium and grow readily. It should therefore be possible to determine the specific requirements of the remaining mutant cultures by selective analysis.

TABLE 4

AUXOTROPHIC MUTANTS OF *H. EUTROPHA*

Strain	Growth Factor Required
A 1	methionine
A 12	histidine
A 14	tryptophan
A 16	methionine
A 23	cystine
A 24	phenylalanine + tryptophan
A 25	histidine

IV A Closed Environment Chemostat for Cultural Stability Studies

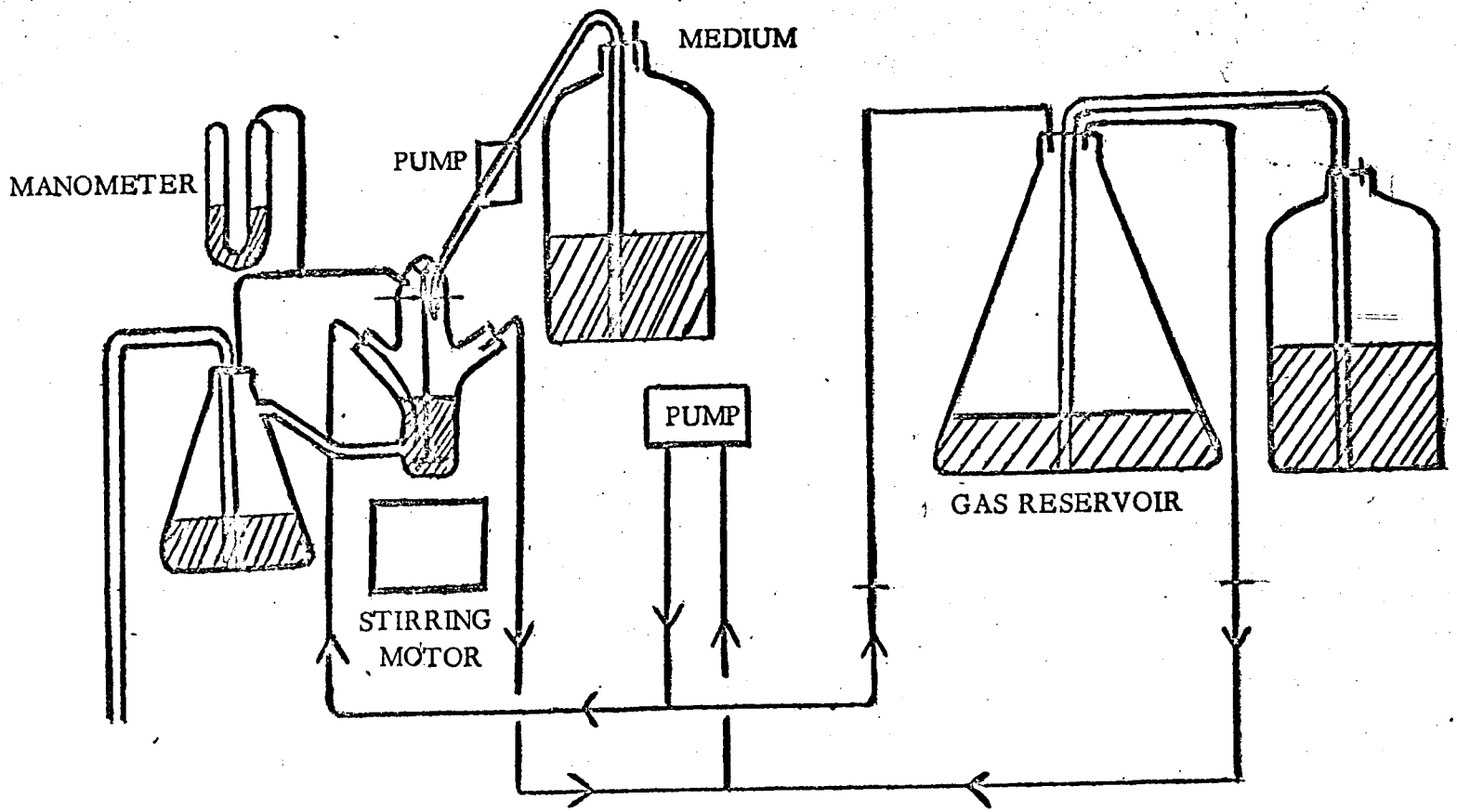
It is well established that a steady-state culture cannot be duplicated by repeated subculturing in broth or on agar. The population dynamics that may be encountered during growth under steady-state conditions are almost certain to escalate, or in some cases, be repressed under the uncontrolled environments afforded by a flask, tube, or plate culture. We have therefore developed a chemostat which will function under a closed recirculating atmosphere and which will allow rapid adjustments to be made in total gas pressure, partial gas pressures of the component gases, and medium flow rate. The system is simple in design and operation, and requires a minimum of time and effort in preparation and maintenance. (Figure 2).

The gas reservoir contains a H_2, O_2, and CO_2 mixture in the desired proportions. The mixture is circulated to and from the culture vessel via a small pressure-vacuum pump with the gas reservoir acting as a circulating by-pass for excess pump pressure. The two clamps leading to and from the gas reservoir enable both the amount of aeration and the gas pressure in the culture vessel to be regulated. A manometer is built into the system to allow the pressure in the culture vessel to be continuously monitored. The culture is aerated via a submerged sparger and a magnetic stirring bar assists in further agitating the suspension. Media is fed from the media reservoir into the culture vessel by means of a peristaltic pump and the flow rate is regulated using a cam timer.

At present the culture vessel being used has a 50 ml capacity but a 250 ml vessel can be substituted if the need arises. The system has undergone a preliminary run with H. eutropha of 7 days duration and after minor adjustments is functioning properly. We intend to observe both normal and mutant cultures of H. eutropha for population changes during prolonged cultivation and then determine the effects of phage infection in this system.

FIGURE 2

CHEMOSTAT FOR CULTIVATION OF HYDROGEN BACTERIA



V Future Research

The effects of the four phage types on autotrophic and heterotrophic cultures will be studied in detail. Of special interest will be the effects of (1) various phage/bacteria ratios and (2) small amounts of organic matter on bacterial susceptibility. The course of phage infection during continious cultivation and the possibility of lysogeny will also be examined.

The metabolism of H. eutropha under simultaneous autotrophic and heterotrophic conditions will be further investigated, especially with regard to CO_2 fixation under these conditions. The nutritional mutants of H. eutropha which are now available will be utilized to correlate the amount and type of organic compound with the degree of activity of the autotrophic system.